VEGF Inhibition and Renal Thrombotic Microangiopathy

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SUMMARY

The glomerular microvasculature is particularly susceptible to injury in thrombotic microangiopathy, but the mechanisms by which this occurs are unclear. We report the cases of six patients who were treated with bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), in whom glomerular disease characteristic of thrombotic microangiopathy developed. To show that local reduction of VEGF within the kidney is sufficient to trigger the pathogenesis of thrombotic microangiopathy, we used conditional gene targeting to delete VEGF from renal podocytes in adult mice; this resulted in a profound thrombotic glomerular injury. These observations provide evidence that glomerular injury in patients who are treated with bevacizumab is probably due to direct targeting of VEGF by antiangiogenic therapy.

The discovery that vascular endothelial growth factor (VEGF) is a critical factor in the growth of blood vessels led to the development of VEGF inhibitors, such as bevacizumab, to treat diseases that are characterized by pathologic angiogenesis. The addition of bevacizumab to chemotherapeutic regimens improved survival rates among patients with cancers of the colon, lung, and breast; it is also used as a single agent for renal-cell carcinoma. With the expanding use of bevacizumab, adverse effects have become apparent. Two of the most common are proteinuria (in 21 to 64% of patients) and hypertension (in 3 to 36%).¹ Nephrotic-range proteinuria, which denotes structural damage to the glomerular

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filtration barrier (Fig. 1A), occurs in 1 to 2% of bevacizumab-treated patients.¹ Although potential causes of this type of proteinuria have been suggested,^{2,3} it has been difficult to distinguish general (off-target) effects of therapy, such as an immunologic response to the monoclonal antibody, from direct (on-target) effects due to inhibition of endogenous VEGF signaling in noncancerous tissues.

This report describes six patients with proteinuria and classic pathological features of thrombotic microangiopathy after bevacizumab therapy. The findings underscore the need for a better understanding of the renal consequences of VEGF inhibition. This topic has particular clinical relevance, given the impressive therapeutic potential of these drugs in a range of cancers and the expectation that an increasing number of patients will receive such agents in the future.

We also provide direct experimental evidence of a mechanism of glomerular injury by VEGF inhibitors in a mouse model, showing that local genetic ablation of VEGF production in the kidney recapitulates the glomerular injury seen in our series of patients. The results support the concept that local production of VEGF plays a critical protective role in the pathogenesis of microangiopathic processes.

CLINICAL CASES

PATIENT 1

A 59-year-old man with hepatocellular carcinoma received bevacizumab as a single agent at a dose of 7.5 mg per kilogram of body weight every 14 days for a total of 24 doses. The therapy led to a reduction in tumor size and a fall in the level of alpha-fetoprotein (from 18,000 to 60 ng per milliliter). The patient's baseline renal function was normal, with minimal proteinuria (urinary protein-to-creatinine ratio, 0.5), but the protein-to-creatinine ratio steadily increased to 3.4 after 9 months. New-onset hypertension requiring triple antihypertensive therapy developed. A renal biopsy showed classic features of thrombotic microangiopathy, with widening of the subendothelial space of glomerular capillaries, duplication of the glomerular basement membranes with cellular interposition, mesangiolysis, and extensive effacement of foot processes (Fig. 1B and 1C). Small arteries and arterioles showed focal endothelial swelling without overt thrombosis. The hematocrit was normal (41%), with a low platelet count (103,000 cells per cubic millimeter) and no schistocytes. After bevacizumab had been discontinued, the patient's hypertension was controlled, and within 3 months, his 24-hour protein excretion was 1.7 g.

PATIENT 2

A 74-year-old man with recurrent hepatocellular carcinoma was treated with bevacizumab as a single agent at a dose of 7.5 mg per kilogram every 2 weeks for a total of four doses. His baseline renal function was normal (serum creatinine, 0.6 mg per deciliter [53 μ mol per liter]), with minimal proteinuria (urinary protein-to-creatinine ratio, 0.4).

After initiation of bevacizumab therapy, proteinuria developed, with a protein-to-creatinine ratio of 1.1 at 1 month, 2.0 at 2 months, and 2.7 at 3 months. Renal biopsy showed classic features of thrombotic microangiopathy, with only focal areas of podocyte foot-process

effacement. The hematocrit was normal (47%), with no schistocytes. After bevacizumab had been discontinued, the proteinuria improved, with a urine-dipstick result of 1+ (30 mg of protein per deciliter) 3 months later.

PATIENT 3

A 56-year-old man with bronchoalveolar carcinoma was treated with cisplatin and gemcitabine, initially with a good response. Because of disease progression 2 years later, bevacizumab was started as a single agent at a dose of 15 mg per kilogram every 3 weeks for a total of 19 doses. Pemetrexed (at a dose of 1000 mg every 3 weeks) was added 7 months later. At baseline, he had normal renal function (serum creatinine, 1.2 mg per deciliter [106 μ mol per liter]) without proteinuria and hypertension.

During treatment, his renal function deteriorated (serum creatinine, 3.1 mg per deciliter [274 μ mol per liter] at 9 months), the hypertension worsened, and minimal proteinuria developed (160 mg of protein per 24 hours). He had anemia (hematocrit, 34%), with a normal platelet count and no schistocytes. Renal biopsy showed thrombotic microangiopathy with focal areas of foot-process effacement. Small arteries and arterioles showed prominent endothelial swelling without overt thromboses. Bevacizumab was discontinued. The patient's status deteriorated rapidly because of his malignant disease, and he died shortly thereafter.

PATIENT 4

A 62-year-old man with a history of type 2 diabetes, hypertension, atrial fibrillation, and chronic kidney disease (serum creatinine, 1.4 mg per deciliter [124 μ mol per liter]) had small-cell lung carcinoma. He was treated with cisplatin and docetaxel, along with bevacizumab at a dose of 10 mg per kilogram every 2 weeks for a total of four doses. Three months later, pneumonia developed, and he was treated with levofloxacin. Shortly thereafter, he was hospitalized with acute renal failure (serum creatinine, 5.7 mg per deciliter [504 μ mol per liter]) and a maculopapular rash. Urinalysis revealed 3+ protein (500 mg per deciliter on a urine-dipstick test) and 3+ blood, with red cells and no casts. He was empirically treated with methylprednisolone and underwent renal biopsy.

The biopsy showed acute thrombotic microangiopathy characterized by mesangiolysis, endothelial swelling, and focal glomerular capillary thrombosis, along with modest mesangial deposition of IgA (Fig. 1B and 1C). Diffuse effacement of foot processes was seen. After the biopsy, bevacizumab was discontinued, and oral corticosteroids were substituted, with a short course of cyclophosphamide. The patient's renal function improved rapidly, and 2 months later, his serum creatinine level was 1.1 mg per deciliter (97 μ mol per liter) and the proteinuria had resolved.

PATIENT 5

A 61-year-old man with metastatic pancreatic cancer was treated with gemcitabine, erlotinib, and bevacizumab, the last at a dose of 10 mg per kilogram every 2 weeks for a total of 12 doses. The baseline creatinine level was 1.0 mg per deciliter (88 μ mol per liter), with no proteinuria. At 5 months, he had generalized edema and decreased urinary output. The serum creatinine level was 2.6 mg per deciliter (230 μ mol per liter), and 24-hour urinary

protein excretion was 4613 mg. A blood smear showed occasional schistocytes with thrombocytopenia (platelet count, 13,000 cells per cubic millimeter; baseline count, 55,000). Bevacizumab was discontinued. Renal biopsy showed classic features of thrombotic microangiopathy with focal areas of podocyte injury. Thrombi were found in some afferent arterioles, leading to ischemic glomerular collapse (Fig. 1 of the Supplementary Appendix, available with the full text of this article at www.nejm.org). After five plasma-pheresis treatments, the patient's renal function stabilized. However, his malignant disease progressed, and he died 1 year later.

PATIENT 6

A 59-year-old woman with metastatic ovarian cancer was treated with paclitaxel and topotecan, without improvement. She was started on bevacizumab as a single agent at a dose of 15 mg per kilogram every 3 weeks for a total of 29 doses. Nine months later, 24-hour urinary protein excretion had increased to 825 mg (baseline level, 235 mg). The serum creatinine level was 0.9 mg per deciliter (80 μ mol per liter). The hematocrit and platelet counts were normal. Renal biopsy showed extensive subendothelial widening, endothelial swelling of glomerular capillary walls with focal mesangiolysis, occasionally fragmented red cells, and mild focal foot-process effacement, findings that were consistent with thrombotic microangiopathy. Sub-endothelial and segmental mesangial deposits were also observed, with IgA predominance. The location of the deposits and the absence of proliferation were not consistent with primary IgA nephropathy. The drug was continued for 8 more months, with persistent proteinuria (900 to 1000 mg of protein per 24 hours) and stable renal function. Bevacizumab was discontinued, and gemcitabine and carboplatin were started. However, the patient died 9 months later.

RESULTS IN AN EXPERIMENTAL MODEL

TIME-SPECIFIC AND CELL-SPECIFIC KNOCKOUT MICE

To determine whether renal thrombotic microangiopathy in patients receiving bevacizumab might be explained by a biologic reduction in glomerular VEGF, we created a relevant experimental murine model that targeted only podocytes, which are the major source of glomerular VEGF production (Supplementary Appendix). We used a conditional expression model (Tet-On system) in which the target gene is deleted only in the presence of a tetracycline derivative. The animals are functionally normal, but when they are exposed to tetracycline, the targeted gene and its protein are eliminated. We used this strategy to delete the VEGF gene in a time-specific manner from podocytes but from no other cell type in mice that were studied at 3, 12, and 24 weeks of age (Fig. 2, and Fig. 2 and 3 of the Supplementary Appendix). The various time points were chosen to ensure that the glomeruli had been fully functional when VEGF was eliminated. Because the features of glomerular injury were equivalent at each of these induction times, our findings refer to the 3-week time point. Before doxycycline was administered to eliminate VEGF, all podocytes expressed VEGF. Deletion of VEGF expression was confirmed by in situ analysis (Fig. 2A).

LOSS OF VEGF FROM ADULT PODOCYTES

Four weeks after induction with doxycycline, 100% of the 62 mutant mice had pronounced proteinuria (1 to 5 g per liter on dipstick testing). In 9 of the mutant mice, the mean ($\pm$ SD) albumin-to-creatinine ratio was 4010 ± 3839 (measured in nanograms per microgram), as compared with 26 ± 14 in 11 controls ($P < 0.001$). Nine weeks after induction, the kidneys of all the mutant mice were pale and shrunken (Fig. 2C), and proteinuria had increased to maximal levels on dipstick testing (5 g per liter), as shown on a sodium dodecyl sulfate–polyacrylamide gel (Fig. 2D).

Four weeks after induction, electron micrographs of glomeruli from VEGF-mutant mice displayed typical features of thrombotic microangiopathy (Fig. 2E). At the onset of proteinuria, podocytes were relatively well preserved (Fig. 2E, and Fig. 4 of the Supplementary Appendix) but appeared abnormal as the disease progressed. Intra-capillary thrombi and bloodless capillary loops that were obliterated by swollen endothelial cells were observed (Fig. 2E). Immunohistochemical analysis was negative for complement components and immune complexes (not shown) but was positive for fibrin (Fig. 2F). Fragmented red cells were observed without thrombocytopenia in 58% of blood smears from seven mutant mice (Fig. 2F).

Blood-pressure levels were normal before the induction of proteinuria (Fig. 5 of the Supplementary Appendix). Five weeks after induction, when glomerular disease was already advanced, VEGF-mutant mice had hypertension, with a mean blood pressure of 129 ± 14 mm Hg, as compared with 113 ± 9.6 mm Hg in controls ($P = 0.004$). In mice that underwent induction at later time points (3 or 6 months), glomerular lesions and hypertension also developed, although the rate of progression was slower. Control mice that received doxycycline did not have glomerular injury, proteinuria, fragmented red cells, or hypertension.

In an attempt to ameliorate the lesions, pharmacologic doses of human VEGF-121 were administered subcutaneously twice daily at a dose of $50 \mu\text{g}$ per kilogram; the dose and preparation were chosen because they have been reported to improve the renal outcome in rats with thrombotic microangiopathy.⁴ This treatment did not improve the outcome or reduce renal injury (Supplementary Appendix).

DISCUSSION

Our findings indicate that the production of VEGF by podocytes is required for health and maintenance of the adjacent glomerular endothelium (Fig. 3). Disruption of VEGF function, through pharmacologic or genetic means, results in a characteristic pattern of renal damage, which suggests that thrombotic microangiopathy in patients who are treated with bevacizumab results from a reduction in glomerular VEGF, a direct, on-target effect of the drug.

A critical role of impaired VEGF signaling within the glomerulus in the pathogenesis of thrombotic microangiopathy is supported by several observations. First, thrombotic microangiopathy in the patients who were treated with bevacizumab was localized in the

kidney, with little or no involvement of the peripheral microvasculature. Second, extrarenal circulating VEGF, which was not affected by our genetic manipulation, did not protect the mutant mice from glomerular thrombotic microangiopathy. Finally, systemic administration of VEGF-121 failed to reduce renal injury in the mice, although higher doses of VEGF-121 might have been beneficial.⁵

Why is the glomerular microvasculature particularly susceptible to VEGF inhibition and thrombotic microangiopathy? Glomerular endothelial cells contain fenestrations that are necessary for the unique permeability characteristics of the glomerular filtration barrier (Fig. 1A).^{6,7} In vitro, VEGF induces the formation of fenestrations in endothelium. We posit that a loss of VEGF from the glomerulus leads to a loss of the healthy fenestrated phenotype and promotes the development of microvascular injury and thrombotic microangiopathy.

Since we eliminated VEGF production only from the podocyte, and since the primary phenotype is observed in endothelial cells across the glomerular basement membrane, our results indicate that VEGF is delivered to the glomerular endothelial cells against the flow of urinary filtrate. Although the mechanism of VEGF transport from podocytes to glomerular endothelial cells is not clear, other investigators⁸ have shown that diffusion across the glomerular basement membrane predominates over convection for molecules smaller than 30 Å; VEGF is about 26 Å. Since podocytes are the major source of VEGF in the glomerulus and produce VEGF constitutively at high levels, there should be a substantial concentration gradient favoring diffusion of VEGF from the podocyte to glomerular endothelial cells. Furthermore, these cells are in proximity (within 200 to 300 nm), and binding of major VEGF isoforms to the glomerular basement membrane has been clearly demonstrated.⁹ Finally, our finding that the loss of podocyte-derived VEGF has profound effects on the adjacent glomerular endothelium provides very suggestive evidence for the existence of this pathway.

In addition to glomerular injury, hypertension developed in mice lacking VEGF in podocytes. Hypertension is reported in up to 36% of patients who are treated with bevacizumab, and it has been suggested that the elevation in blood pressure leads to proteinuria and glomerular disease.¹⁰ In our murine model, glomerular injury preceded hypertension, indicating that elevated blood pressure cannot be the initial trigger for thrombotic microangiopathy in this setting. On the other hand, high blood pressure without accompanying proteinuria is seen in a substantial proportion of bevacizumab-treated patients, suggesting that inhibition of VEGF may induce hypertension through diverse mechanisms.

A consensus view on the consequences of VEGF inhibition in the mature kidney has yet to emerge from studies in animals. The administration of VEGF-blocking antibodies or an adenovirus-expressing sFlt (soluble fms-like tyrosine kinase) in rodents caused a clinical syndrome with features of preeclampsia, including endotheliosis, proteinuria, and hypertension.^{11,12} In contrast, the administration of blocking VEGF aptamers had no effect on healthy adult rats.¹³ In preclinical safety trials of bevacizumab in nonhuman primates, there were no reported adverse renal effects.¹⁴ However, the methods for determining the extent and location of VEGF inhibition, possible nonspecific effects that may be

independent of antiangiogenic actions, and failure to use rigorous and sensitive measures of renal injury may account for these conflicting results. Our strategy was to use conditional genetic targeting to ablate VEGF production specifically from a defined cellular compartment within the mature glomerulus without affecting VEGF levels in any other tissues. This approach obviates concern about confounding effects related to VEGF requirements during glomerular development, allows for accurate and direct assessment of the extent of local VEGF knockdown, and shows definitively that local, ongoing VEGF production by podocytes is necessary for the functioning of the adult glomerular filtration barrier.

In contrast to the murine model, which causes an irreversible and virtually complete loss of VEGF production in podocytes, the effects of bevacizumab are transient in humans. In our patients, renal function, proteinuria, and blood pressure improved when the drug was withdrawn, suggesting that these processes are reversible. This course is similar to that seen in preeclampsia, in which increased levels of sFlt bind and inactivate both VEGF and placental growth factor.^{11,15,16} Preeclampsia typically resolves after delivery of the placenta, the source of excess sFlt.

Clinical reports suggest that many patients may have increased protein excretion during treatment with bevacizumab, yet few of them have nephrotic-range proteinuria. The identification of factors that confer susceptibility to overt glomerular disease in this subgroup of patients will be important. Because altered glomerular permeability appears to be a direct consequence of VEGF inhibition, proteinuria may correlate with drug efficacy — a relationship that could be examined in future clinical studies.

Although the incidence of kidney injury among patients receiving VEGF inhibitors is not known, our data suggest that it may be prudent to monitor patients receiving VEGF inhibitors closely for possible kidney injury. The optimal way to monitor such patients is not known. Although there have been sporadic reports of other glomerular lesions associated with VEGF-inhibitor therapy, our findings in six patients, together with three previous case reports of renal thrombotic micro-angiopathy,^{17–19} suggest that this pathological lesion may be typical when profound changes in renal function are observed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Microangiopathy in Patients Who Were Treated with Bevacizumab

Panel A shows the ultrastructure of a healthy glomerular filtration barrier, which is composed of three layers: the outermost podocyte layer, the fenestrated glomerular endothelial cells, and an intervening glomerular basement membrane. Urinary filtrate passes from the blood lumen to the urinary space. Podocytes produce vascular endothelial growth factor (VEGF). The tyrosine kinase receptors for VEGF (VEGFR-1 and VEGFR-2) are expressed by glomerular endothelial cells. A slit diaphragm is indicated by the arrow, and fenestrations are indicated by arrowheads. (Photomicrograph courtesy of Dr. Wilhelm Kriz, Mannheim, Germany.) Panel B shows silver staining of two representative glomeruli in biopsy specimens from patients. In a specimen from Patient 4 (left), mesangiolysis (single arrow), prominent endothelial swelling (arrowhead), red-cell fragments (double arrows), and thrombi are visible in some capillary loops. In a specimen from Patient 1 (right), the double contours of capillary basement membranes (arrows) can be seen. Panel C shows

transmission electron micrographs of glomeruli from Patient 4 (left), revealing fibrillar material that is characteristic of fibrin (arrows), and from Patient 1 (right), revealing duplication of capillary basement membranes (arrow) and a marked widening of the subendothelial spaces by electron-lucent material (arrowheads).

Figure 2. Thrombotic Microangiopathy Caused by Genetic Deletion of VEGF from Glomeruli in a Murine Model

In Panel A, the successful excision of vascular endothelial growth factor (VEGF) from podocytes was confirmed by a lack of VEGF RNA expression after induction with doxycycline (+Dox), as compared with normal levels of Wilms' tumor suppressor 1 (WT-1) RNA, another gene expressed by podocytes. Results from a control mouse (–Dox) are shown for comparison. Panel B shows the breeding strategy that was used to generate a time-specific and cell-specific knockout of VEGF in podocytes. In the absence of doxycycline (yellow circles), the reverse tetracycline transactivator protein (rtTA, red crescents) cannot bind to the tetracycline responsive element in the tetO-Cre transgene. In the presence of doxycycline, rtTA, under control of the podocyte-specific promoter (podocin), binds to initiate transcription of Cre recombinase specifically in podocytes. In Panel C, a kidney from a +Dox mouse 9 weeks after induction with doxycycline is pale, small, and sclerotic, findings that are consistent with end-stage kidney failure, as compared with a normal kidney from a –Dox mouse. As shown in Panel D, albuminuria was detected 4 weeks after induction in all mutant mice (arrow). All control (Con) and mutant (KO) mice received doxycycline. Molecular mass in kilodaltons is shown on the left. L denotes ladder, the molecular reference for protein size. In Panel E, in glomeruli that were stained with

silver methenamine outlining basement membranes (subpanels a and b), the lumens of glomerular capillaries that are seen in control mice (subpanel a) are either obliterated or collapsed in mutant mice (subpanel b). Electron micrographs (EM) showing the ultrastructure of glomeruli (subpanels c, d, and f) reveal swollen endothelial cells (arrow, sub-panel d) and dense subendothelial deposits (white arrows, subpanel f) in capillary loops of VEGF mutants. Fenestrated endothelium (arrowheads, subpanel c) is shown in controls for comparison. Podocyte foot processes are relatively spared early in disease (white arrowheads, subpanel f). On light microscopy, Martius scarlet blue (MSB) staining shows an intracapillary thrombus in a mutant mouse (double arrows, subpanel e). In Panel F, schistocytes (arrows) were found in blood smears from 58% of mutant mice (subpanel a). Immunohistochemical staining for fibrin of glomeruli from VEGF mutants shows positive results (reddish color in subpanel b). There was no staining in glomeruli of control littermates (not shown).

Figure 3. Hypothetical Model of Disruption of VEGF Signaling in Renal Thrombotic Microangiopathy

The loss of function of vascular endothelial growth factor (VEGF) through genetic deletion (VEGF KO), pharmacologic inhibition, or an elevated level of circulating soluble fms-like tyrosine kinase 1 (sFlt-1) that binds VEGF is associated with damage to the glomerular endothelium characterized by swelling and thrombotic microangiopathy. VEGFR-2 denotes kinase insert domain receptor.